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(71) Applicant: CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US).

(72) Inventors: GROVES, Eric, S. ; 546 Morecroft Road, Lafayette, CA 94549 (US). ALDWIN, Lois ; 179 Lakeshore Drive, San Mateo, CA 94402 (US). WINKELHAKE, Jeffrey, L. ; 1400 Fifty-Third Street, Emeryville, CA 94608 (US). NITECKI, Danute, E. ; 2296 Virginia Street, Berkeley, CA 94709 (US). GAUNEY, Stacey ; 23150 Lakeridge Avenue, Hayward, CA 94541 (US). RUDOLPH, Alfred, A. ; 258 Claudia Court, Moraga, CA 94556 (US).

(74) Agent: HALLUIN, Albert, P.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US).

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(57) Abstract

Compositions and process for preparing polymer-antibiotic conjugates are disclosed. The polymer is conjugated to the antibiotic via a covalent bond. Specific polymers that are useful in the present invention include polyalkalene glycols, copolymers of different polyalkalene glycols, polyvinylpyrrolidone, polyvinyl alcohol, homopolymers of amino acids, cellulose and cellulose derivatives, starch and starch derivatives. Preferred antibiotics are those which have a low therapeutic index.

* See back of page

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Polymer/Antibiotic Conjugate

The present invention relates to a polymer-antibiotic conjugate. More specifically, the present invention relates to a polymer-antibiotic conjugate that has more beneficial properties than the antibiotic alone.

Antibiotics have an obvious utility in treating various diseases caused by infectious agents. However, some antibiotics are less preferred because they exhibit toxic side effects at therapeutic doses or because their in vivo half-life is too short. Because of these limitations, their therapeutic effectiveness is greatly diminished. For example, they may be limited to topical use even though they demonstrate in vitro activity against various human internal pathogens. This limitation can frustrate therapeutic treatment because these antibiotics can be the only known, or the most effective, agents to combat these internal pathogens.

The antibiotics that have toxic side effects and a short in vivo (circulating) half-life are known to those of ordinary skill in the art. They are listed in the general literature that is distributed to physicians, as well as in articles in scientific publications. Some of them are discussed below.

In other areas of therapeutic research, it has been found that the attachment of polymers to various compounds can provide a benefit when those compounds are used parenterally. For example, U.S. Patent No. 4,179,337 to Davis et al. disclose conjugating polyethylene glycol (PEG) to various proteins to reduce their immunogenicity, U.S. Patent No. 4,766,106 to Katre et al. discloses conjugating polymers to lipophilic proteins to increase their solubility and circulating half-life, etc. (see also U.S. Patent Nos. 4,412,989, 4,495,285, 4,496,689, 4,261,973, 4,609,546, and 4,732,863 which disclose conjugation of polymers to various other proteins for a variety of purposes; all of these U.S. patents are hereby incorporated by reference in their entireties).

Also, polymer conjugation has been reported with non-protein compounds. For example, Ferruti et al., Makromol. Chem. 182:2183-2192 (1981) and Ulbrich et al., Makromol. Chem. 187:1131-1144 (1986) both disclose conjugating PEG to drug models. Ouchi et al. have reported conjugates of PEG to 5-Fluorouracil (see J. Poly. Sci.-Part C: Polymer Letters 25:279-285 (1987) and J. Macromol. Sci.- Chem. A24(9):1011-1032 (1987)). Batz, Adv. Poly. Sci. 23:25-53 (1977) and Samour, Chemtech 494-501 (August 1978) summarize work performed by researchers which disclose polymers and antibacterials, such as tropolone, penicillin, nitrofurans, and sulfanilic amide. However, the antibacterials were either formed into polymers themselves, or they were multiply derivatized to one polymer molecule. In the multiply

derivatized state, these antibiotic molecules are slowly released after the bond between the polymer and the antibacterial is cleaved. The purpose of slow release is to avoid a toxic bolus of the drug.

Zaffaroni et al., review a reference in which the authors created a dextran-kanamycin complex which was primarily designed to exhibit its activity when the kanamycin was cleaved from the dextran. This reference is Snezhko et al., Antibiotiki (Moscow) 17,48 (1972) and it also states that tetracycline and ampicillin were used and that the type of chemical bonds between the antibiotic and the dextran had a significant effect (ie. sometimes a significant decrease) on the antimicrobial activity. Zalipsky et al., Eur. Polym. J. 19:1177-1183 (1983) disclose conjugating PEG to drugs of different classes using several conjugation techniques. The drugs that are conjugated to PEG include penicillin V, aspirin, amphetamine, quinidine, and atropine. Zalipsky et al. also report that others have conjugated penicillin to polymers (see Samour, supra.; Ushakov et al., Vysokomolek. Soedin. 282 (1964); and Ushakov et al., Dokl. Akad. Nauk. SSSR 149:334 (1963)). However, some of the antibiotics disclosed in Snezhko et al. and Zalipsky et al. already have a high therapeutic index. Specifically, tetracycline, ampicillin, and penicillin are all safe to use and conjugation would not substantially increase their therapeutic index. Furthermore, the complexes disclosed in Snezhko are less effective if they are covalently conjugated for a substantial period of time (the activity goes down with more stable bonds). This implies that covalent conjugation is detrimental and that the activity derives from hydrolysis and release of free antibiotic. U.S. Patent No. 4,310,397 to Kaetsu et al. discloses polymers containing a physiologically active substance. Kaetsu et al. state that the active substances are entrapped within the polymer when they are added to polymerizable monomers and subjected to ionizing radiation. Polymerizable monomers can include polyethylene glycol methacrylate and the active substances can include antibiotics. All of the previously mentioned references are hereby incorporated by reference in their entireties.

However, none of the aforementioned references specifically discloses covalently conjugating polymers to antibiotics so that the conjugates are stable for a substantial period of time, to increase their therapeutic index, to reduce the adverse side effects of these antibiotics, and to increase their circulating half-life. Accordingly, the present invention is directed at solving these problems.

The present invention is a biologically effective polymer-antibiotic conjugate comprising at least one biologically compatible polymer conjugated to one antibiotic molecule through a covalent bond that is stable for a substantial period of time, wherein the therapeutic index of the conjugate is substantially greater than that of the

free antibiotic and the activity of the conjugate is substantially the same as the free antibiotic. Preferably, the polymers are selected from the group consisting essentially of polyalkylene glycols, copolymers of different polyalkylene glycols, polyvinylpyrrolidone, polyvinyl alcohol, homopolymers of amino acids, cellulose and cellulose derivatives, heparin, starch and starch derivatives. More preferably the polymer is polyethylene glycol (PEG). Preferably, the antibiotics are selected from the group consisting essentially of aminoglycosides; β-lactams; chloramphenicol and its derivatives; lincosaminides; macrolides; nucleosides; oligosaccharides; peptides; polyenes; and tetracyclines; sulfonamides, nitrofurans, and quinolone carboxylic acids. More preferably the antibiotics are peptides or polyenes, such as polymyxin or amphotericin respectively.

Among other factors, the present invention is based on the discovery that an antibiotic with a low therapeutic index (for example an antibiotic that is toxic to the kidney at normally therapeutic dose levels, such as polymyxin or amphotericin) can be covalently conjugated to a polymer and still retain substantial antimicrobial activity of the free antibiotic. Also, it is believed that the antibiotic's therapeutic index can be significantly improved by conjugating it to a polymer. For example, a polymer-polymyxin conjugate could conceivably reduce the nephrotoxicity that is caused by polymyxin alone. Also, the conjugate may persist in the body for a longer period of time (due to its size, resistance to degradation by metabolic anzymes, or other factors), thereby increasing the circulating half-life and reducing the total dose needed for efficacy. It is also believed that conjugation may change the specific activity or increase/decrease solubility of the antibiotic.

Furthermore, it is believed that the present polymer-antibiotic conjugate is bioactive in the conjugated form. This is unlike many of the prior art references which describe polymers of antibiotic molecules or polymer molecules that are multiply derivitized with antibiotic molecules so that the active maolecules of antibiotics can be individually and slowly released. These forms are not necessarily bioactive as conjugates.

More specifically, the present invention comprises polyethylene glycol covalently conjugated to polymyxin or amphotericin. Polyethylene glycol is conjugated to polymyxin or amphotericin by a urethane, ester, or amide bond.

Figure 1 is an elution profile of a reaction mixture of PEG-pNP and polymyxin containing PEG-polymyxin and various compounds on a G-50 Sephadex size exclusion column.

Figure 2 is an elution profile of peak A shown in Figure 1 from the G-50 Sephadex column shown in Figure 1 on S-Sepharose.

Among other things, the present invention relates to polymer-antibiotic conjugates, the process of their manufacture, and the process of their use.

The Polymer

"Polymer" is defined as a water soluble compound having a structure characterized by repeating units. The polymer is biocompatible, meaning that it is safe when parenterally administered, and preferably it is non-toxic and non-immunogenic in humans. These repeating units may be small groups of saturated or unsaturated hydrocarbons containing oxo or hydroxy groups, amino acids, or larger groups which may include ring structures. Preferred polymers are polyalkylene polyols, such as: polyoxyethylated glycerol; polyoxyethylated sorbitol; polyoxyethylated glucose; polyethylene glycol (PEG); propylene glycol; and copolymers of propylene glycol and ethylene glycol. Other preferred polymers are: polyvinylpyrrolidone; polyvinyl alcohol; homopolymers of amino acids, such as polyproline; sulfated sugars, such as heparin; cellulose and cellulose derivatives; and starch or starch derivatives. Many of these polymers have been parenterally used or are related to naturally occurring molecules so that it is expected that the polymer would not stimulate an immune response. Preferred polymers, conjugation processes, and purification processes are shown in the following U.S. Patents and European Patent Applications which are hereby incorporated by reference in their entireties: Hiratani, U.S. Patent No. 4,609,546; Iwashita et al., U.S. Patent No. 4,412,989; Katre et al., U.S. Patent No. 4,766,106; Davis et al., U.S. Patent No. 4,179,337; Lee et al., U.S. Patent No. 4,261,973; Mitra, U.S. Patent No. 4,496,689; Shimizu et al., U.S. Patent No. 4,495,285; European Patent Application No. 236,987 to Takeda; European Patent Application No. 183,503 to Beecham; Japanese Patent Application No. 62-185029 to Ajinomoto; and Japanese Patent Application No. 62-252800 to Vitamin Kenkyusho. PCT/US89/00270, Knauf et al., J. Bio. Chem. 263:15064-15070 (1988), Zalipsky et al., Eur. Polym. J. 19:1177-1183 (1983), and Veronese et al., App. Biochem. and Biotech. 11:141-152 (1985) are also hereby incorporated by reference in their entireties.

Preferably, the polymers have at least one reactive group so that they can be conjugated to a free amino or carboxylic acid group on the antibiotic, preferably this group is a free hydroxyl group . When the polymer has more than one free hydroxyl group, as does PEG, it may be preferable to substitute it with an organic group having from 1 to 5 carbon atoms. Preferably, the organic group is an alkyl group. More preferably, it is a methyl group.

The preferred molecular weights of the polymers recited above are between 600 and 20,000, more preferably the polymers have molecular weights between 2,000 and

12,000. However, when polymers are conjugated to molecules, such as proteins or antibiotics, the hydrodynamic radius of the conjugate appears larger than what would be predicted based on molecular weight of globular proteins. It is preferred that the hydrodynamic radius of the polymer-polymyxin conjugate is large enough to resist filtration through the kidney. Hydrodynamic radius is defined as the effective molecular radius of a particle in an aqueous environment. Albumin has a molecular weight of 68 Kd and is typically too large to filter through the kidney. See Knauf et al. supra for information on the relationship of effective molecular size to clearance rate. Additionally, it is anticipated that these conjugates will have an altered biodistribution because of their hydrophilic/hydrophobic properties. The new biodistribution may reduce toxicity and enhance antibiotic function by focusing the conjugate at the sites of inflammation and by improving the entrance rate into membranes of bacteria, fungi, or other cells.

Antibiotics

Antibiotics are chemical substances which inhibit the growth of microorganisms, such as bacteria and fungi. Many are typically isolated from naturally occurring microorganisms (i.e., Actinomycetes), but can also be chemically synthesized. Of the many antibiotics that have been isolated and identified, only a small number are sufficiently nontoxic to be medically useful.

Antibiotics can be classified by the way they inhibit cell growth. For example, they can interfere with: cell wall synthesis; cell membrane synthesis or function; protein synthesis; nucleic acid metabolism; and intermediate metabolism. To inhibit protein synthesis, nucleic acid metabolism, and intermediate metabolism, the antibiotic must penetrate and cross the cell membrane and wall. This may be difficult when the antibiotic is covalently conjugated to a polymer thus making the conjugate larger than the antibiotic alone. However, some polymers may actually facilitate transfer across the membrane and cell wall due to their nonionic properties, because they stimulate pinocytosis, or due to receptor mediated endocytosis. Some antibiotics do not cross cell membranes, but exert their activity by changing the membrane properties; such antibiotics may retain their activity while conjugated to polymers.

Antibiotics can also be grouped by the chemical structure. For example, some antibiotics are, or contain, β-lactam rings (penicillins), amino sugars in glycosidic linkage (aminoglycosides), macrocyclic lactone rings (macrolides), polycyclic derivatives of napthacenecarboxamide (tetracyclines), nitrobenzene derivatives of dichloroacetic acid, peptides (bacitracin, gramicidin, and polymyxin), large rings with a conjugated double bond system (polyenes), sulfa drugs derived from sulfanilamide

(sulfonamides), 5-nitro-2-furanyl groups (nitrofurans), quinolone carboxylic acids (i.e., nalidixic acid), and many others.

Antibiotics that are prefered in the present invention are those that have a low theraputic index. Theraputic index is defined as the median lethal dose (LD_{50}) to the median effective dose (ED_{50}). It had been defined as the maximum tolerated dose to the minimum curative dose, but was redefined due to the variability in individual responses
(see, Dorland's Illustrated Medical Dictionary, 27th Ed.,W. B. Saunders Co. (1988)). Antibiotics which have low theraputic indexes can be found in references such as Zinsser Microbiology, 17th edition, W. Joklik et al.- Editors, page 235-277 (1980) or Encyclopedia of Chemical Technology, 3rd edition, Kirk-Othmer- Editors, Volume 2, pages 782-1036 (1978) and Volume 3, pages 1-78, which are hereby incorporated by reference in their entireties, or other standard texts which discuss antibiotics. These references disclose many antibiotics that are useful in preparing the present conjugates. Some of the antibiotics that are disclosed in these references, and are recited here, are safe in their free form, however they may have derivatives which have a low theraputic index and these derivatives would be prefered in the present invention. Preferred antibiotics are those which can be conjugated to polymers through free amine, hydroxyl, sulfhydryl, or carboxyl groups. It is also preferred that these antibiotics have molecular weights between 100 and 5,000 . Without wishing to be bound by theory, it is believed that polymer conjugation can substantially increase the theraputic index of the free antibiotic. Conjugation may also increase antibiotic solubility, lower the antibiotic's toxicity to specific organs (ie. if a conjugate is too large to pass through the glomerulus, the antibiotic cannot exert a toxic effect on certain kidney cells, as polymyxin does in the nephrons), and increase the antibiotic's circulating half-life (which can also decrease the toxic side effects because less antibiotic is necessary).

The groups of antibiotics mentioned above are examples of preferred antibiotics, examples of antibiotics within those groups are: peptide antibiotics, such as amphomycin, bacitracin, bleomycin, cactinomycin, capreomycins, colistin, dactinomycin, enduracidin, gramicidin A, gramicidin J(S), mikamycins, polymyxins, stendomycin, thiopeptin, thiostrepton, tyrocidines, viomycin, virginiamycins, and actinomycin (see Kirk-Othmer, Volume 2 at page 991); aminoglycosides, such as streptomycin, neomycin, paromomycin, gentamycin, ribostamycin, tobramycin, amikacin, and lividomycin (see Kirk-Othmer, Volume 2 at pages 8-19); β-lactams, such as benzylpenicillin, methicillin, oxacillin, hetacillin, piperacillin, amoxicillin, and carbenicillin (see Kirk-Othmer, Volume 2 at page 871); chloramphenicol (see Kirk-Othmer, Volume 2 at page 920); lincosaminides, such as clindamycin, lincomycin

celesticetin, desalicetin (see Kirk-Othmer, Volume 2 at page 930); macrolides, such as erythromycin A-E, lankamycin, leucomycins , and picromycin (see Kirk-Othmer, Volume 2 at page 937); nucleosides, such as 5-azacytidine, amicetin, puromycin, and septacidin (see Kirk-Othmer, Volume 2 at page 962); oligosaccharides, such as curamycin, and everninomicin B (see Kirk-Othmer, Volume 2 at page 986); phenazines, such as, myxin, lomofungin, iodinin, etc. (see Kirk-Othmer, Volume 3 at page 1); polyenes, such as amphotericins, candicidin, nystatin, etc. (see Kirk-Othmer, Volume 3 at page 21); polyethers (see Kirk-Othmer, Volume 3 at page 47); tetracyclines, such as chlortetracycline, oxytetracycline, demeclocycline, methacycline, doxycycline, and minocycline (see Kirk-Othmer, Volume 3 at page 65); sulfonamides, such as sulfathiazole, sulfadiazine, sulfapyrazine, and sulfanilamide (see Kirk-Othmer, volume 2, page 795); nitrofurans, such as nitrofurazone, furazolidone, nitrofurantoin, furium, nitrovin, and nifuroxime (see Kirk-Othmer, volume 2, page 790); quinolone carboxylic acids, such as nalidixic acid, piromidic acid, pipemidic acid, and oxolinic acid (see Kirk-Othmer, volume 2, page 782). Not all of these antibiotics have low theraputic indexes, however persons skilled in the art may choose those antibiotics which do have low indexes to improve their overall theraputic utility. The other listed antibiotics may still be conjugated to the present polymers, however the conjugation will not necessarily increase the theraputic index.

Polymyxin

One of the specifically preferred antibiotics is polymyxin. As used herein, "polymyxin" includes polymyxins A, B, C, D, and E, modifications, analogues thereof, and like compounds (see The Merck Index, Tenth Edition, M. Windholz - Editor, published by Merck and Company, and The Pharmacological Basis of Theraputics, Seventh Ed., Macmillan Publishing (1985) for reference to polymyxin compounds and polymyxin purification processes, both of which are hereby incorporated by reference in their entireties). One of the most common polymyxins is Polymyxin B. It is one of several polymyxin antibiotics produced by various strains of Bacillus polymyxa ; however, the above definition also includes polymyxins that are made by other methods. Polymyxin can be administered as a sulfate salt. It is reported that polymyxin B sulfate is a white, hygroscopic powder, which is relatively odorless. Polymyxin B sulfate is freely soluble in water, soluble in physiological saline, and slightly soluble in alcohol.

Polymyxin has the general structure shown at pages 352 and 1093 of the Merck Index and at pages 1006 and 1008 of Kirk-Othmer, Volume 2. It has five free amine groups which can be conjugated to the polymer. Without wishing to be bound by

theory, it is believed that the polymer is conjugated to one or more of these free amine groups.

Polymyxin is a bactericidal antibiotic. It binds to lipid phosphate groups in the bacterial cytoplasmic membrane and causes leakage through the membrane. Polymyxin's spectrum of activity is similar to colistin derivatives. It is active in vitro against many gram negative organisms; however, most Proteus and Neisseria species are resistant, as are all gram positive and fungi. Polymyxin will inhibit most Escherichia coli, Hemophilus influenzae, Enterobacter aerogenes, Klebsiella pneumoniae, and Pseudomonas aeruginosa. It is typically used to treated acute infections of the urinary tract or meninges, septicaemia, ocular infections, infections of the gastrointestinal tract, and to prevent bacteriuria and bacteremia. It may have utility in treating septic shock as polymyxin can bind lipopolysaccharide (LPS) and block LPS activity in vitro.

The serum half-life of polymyxin B is reported to be 4.3 to 6 hours in adults with normal renal functions and in patients with creatinine clearance of less than 10 mL/minute. Approximately 60% of this dose is excreted through the kidney into the urine by glomerular filtration. Excretion continues for 24 to 74 hours after the final dose. Nephrotoxicity and neurotoxicity are the most serious adverse effects of parenteral polymyxin B sulfate therapy and are most likely to occur in patients receiving higher than recommended doses and in patients with impaired renal function. Nephrotoxicity can be shown by albuminuria, cylindruria, azotemia, hematuria, leukocyturia, excessive electrolyte excretion, and high blood concentrations of polymyxin without a concomitant increase in dosage. The usual intravenous dosage is 15,000 to 25,000 units per kilogram daily with adult patients having normal renal functions.

Polymyxin B sulfate is reconstituted from a powder by dissolving the drug in 5% dextrose with sterile water or physiological saline. The following references provide further data for polymyxins and are hereby incorporated by reference in their entireties; The Merck Index, Tenth Edition, M. Windholz - Editor, published by Merck and Company, and Drug Information - 88, Published by the American Society of Hospital Pharmacists.

Amphotericin

Amphotericin is also a specifically preferred antibiotic in the present invention. As used herein, "amphotericin" includes the different forms of amphotericin, modifications, analogs thereof, and like compounds. It is a polyene macrolide antifungal antibiotic (see Kirk-Othmer, volume 3 at page 21, Zinsser at page 245,

Merck at page 85, and Drug Information-88 at page 71). It is typically produced by Streptomyces nodosus. It appears as a yellow to orange powder that is relatively odorless and insoluble in water and anhydrous alcohol. When it is used parenterally, it is solubilized with sodium deoxycholate for aqueous administration.

The structure of amphotericin is shown in Mechlinski et al., Tetrahedron letters 44: 3873-3876 (1970) Pergamon Press; Mechlinski et al. Jour. of Antibiotics 15:256-258 (1972); Kirk-Othmer; Merck; and other references. It has an amino sugar attached in a 38 membered lactone ring. Without wishing to be bound by theory, it is believed that the polymer can be conjugated to this amino group.

Amphotericin typically inhibits the following fungi and protozoa: Aspergillus fumigatus, Paracoccidioides brasiliensis, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, Mucor mucedo, Rhodotorula spp., Sporothrix shenckii, Blastomyces dermatitidis, Candida spp., Leishmania spp., Naegleria spp., and Acanthamoeba spp. This antibiotic is administered parenterally to treat systemic infections and meningitis caused by fungi. It can also be administered for the treatment of cutaneous or mucocutaneous protozoan infections. However, the following side effects may be observed such as: headache, chills, fever, malaise, muscle and joint pain, anorexia, weight loss, dyspepsia, cramping, epigastric distress, nausea, vomiting, nephrotoxicity, cardiac enlargement, hypokalemia, and anemia.

Process For Preparing And Formulating The Conjugate

There are many different ways to conjugate the polymer to the antibiotic. Typically, the polymer is first activated and then reacted with the antibiotic. Activation is the process of attaching a group to the polymer which will have affinity for the antibiotic and will covalently bond to it. Processes for activating polymers are shown in the patents and applications which are incorporated by reference above. Preferred conjugation processes are shown in Katre et al., Iwashita et al., PCT/US89/00270, Japanese Patent Application No. 62-185029, Japanese Application No. 62-252800, Zalipsky et al., and Veronese et al. The polymer is conjugated to the antibiotic through a covalent bond that is stable for a substantial period of time. Without wishing to be bound by theory, we believe that spontaneous release of the antibiotic does not occur in the serum and that the bioactivity is present when the antibiotic and the polymer are joined through a covalent bond . Preferably, from one to four polymers are attached to polymyxin, more preferably from one to two. Preferably, one polymer is attached to amphotericin. The number of polymers conjugated to other antibiotics will depend on the number of amine or carboxylic acid groups on the antibiotic and the effect that the number has on bioactivity. As shown in the incorporated references, the bond between

the polymer and the polymyxin can be a urethane, an amide, or an ester bond, for example.

Generally the process involves preparing an activated polymer and thereafter reacting the antibioti6 with the activated polymer. Typically, the reaction is carried out in a buffer of pH about 7-9, preferably at about pH 7.5. Preferably, the reaction is carried out generally at 0 to 25°C and from about 20 minutes to about 12 hours; more preferably, the reaction is carried out at 25-35 minutes at 20°C or three hours at 4°C. Following the conjugation, the desired product is recovered and can be purified.

The modification reaction with active PEG can be performed in many ways using one or more steps. Examples of suitable modifying agents that can be used to produce the activated PEG in a one-step reaction include cyanuric acid chloride (2,4,6-trichloro-S-triazine).

In one preferred embodiment the modification reaction takes place in two steps wherein the PEG-OH is reacted first with an acid anhydride such as succinic or glutaric anhydride to form a carboxylic acid, and the carboxylic acid is then reacted with a compound capable of reacting with the carboxylic acid to form an activated PEG with a reactive ester group that is capable of reacting with the protein. Examples of such esters include N-hydroxysuccinimide, sulfo-N-hydroxysuccinimide, 4-hydroxy-3-nitrobenzene sulfonic acid, ortho- or para-nitro phenols, and the like. Preferably, N-hydroxysuccinimide is used.

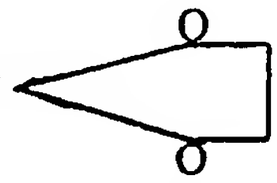
As an example of this process, monomethyl PEG (mPEG) may be reacted with glutaric anhydride at elevated temperatures, preferably about 100-110°C, for four hours. The monomethyl PEG-glutaric acid thus produced is then reacted with N-hydroxysuccinimide in the presence of a carbodiimide reagent such as dicyclohexyl or diisopropyl carbodiimide to produce the activated polymer, methoxypolyethylene glycol-N-succinimidyl glutarate. This compound can then be reacted with the protein after it has been purified. This method is described in detail in Abuchowski et al., Cancer Biochem. Biophys. 7:175-186 (1984), which is hereby incorporated by reference in its entirety.

In another example, mPEG is reacted with glutaric anhydride, then with 4-hydroxy-3-nitrobenzene sulfonic acid (HNSA) in the presence of dicyclohexyl carbodiimide to produce the activated polymer. HNSA is described in Bhatnagar et al., Peptides: Synthesis-Structure-Function, Proceedings of the Seventh American Peptide Symposium, Rich et al. (eds.) (Pierce Chemical Co., Rockford, IL, 1981), p. 97-100, in Nitecki et al., High-Technology Route to Virus Vaccines (American Society for Microbiology: 1985), p. 43-46 (based on talk November 8-10, 1984), entitled "Novel Agent for Coupling Synthetic Peptides to Carriers and Its Application", and in Aldwin

et al., Anal. Biochem. (1987) 164:494-501. All of these disclosures are incorporated herein by reference.

As ester bonds in the final product are chemically and physiologically more reactive than amide bonds, it may be preferable to derivatize the protein with activated polyethylene glycol molecules that would not generate esters in the final product.

In one embodiment, the PEG may be activated using PEG-amine or PEG-OH as starting materials. The PEG-OH may be converted to the PEG-amine as described by V.N.R. Pillar et al., J. Organic Chem. 45:5364-5370 (1980), the disclosure of which is incorporated herein by reference. Briefly, mPEG-amine is prepared by converting mPEG-OH to mPEG-tosylate and then to mPEG-phthalimide, and the phthalimide is cleaved with hydrazine to produce mPEG-NH_2 in a Gabriel synthesis. The mPEG-amine is then reacted with glutaric anhydride at room temperature for about four hours to produce mPEG-$NHCO(CH_2)_3COOH$. After the reaction the product is precipitated, purified, and reacted with N-hydroxysuccinimide and

dicyclohexylcarbodiimide to produce mPEGNHCO$(CH_2)_3$COO-N 

This compound can then be reacted with the appropriate free amino group(s) on the antibiotic (for example) and the product will have no internal ester.

In another embodiment, active ester forms of PEG carboxylic acid useful for such conjugation are described in Nitecki et al., Peptide Chemistry 1987, Shiba & Sakakibara (Ed). Protein Research Foundation, Osaka (1988). Briefly, the active esters have a formula of:

$$R\text{-}(O\text{-}CH_2\text{-}CH_2)_n\text{-}O\text{-}(CHR^2)\text{-}CO\text{-}OLG \quad (I) \text{ or}$$

$$R\text{-}(O\text{-}CH_2\text{-}CH_2)_{n\text{-}1}\text{-}O\text{-}CH_2\text{-}CO\text{-}OLG \quad (II)$$

wherein R is a lower alkyl group of 1-4 carbon atoms, R^2 is H or an organic substituent, n is about 8-500, LG is a leaving group selected from cyanomethyl, an aromatic group selected from a phenyl or naphthyl group substituted with from 1 to 5 substituents that render the aromatic group more labile, and a pyridyl group optionally containing 1-4 of these substituents. For Compound I, these esters may be produced by alkylating PEG with an alpha-haloalkanoic acid or ester thereof followed by esterification with HO-CH_2-CN or the group corresponding to LG, or, for Compound (II), by oxidation of the PEG to its acid, followed by esterification with HO-CH_2-CN or the group corresponding to LG. Most preferably, Formula I is prepared and the activating agent is para-nitrophenol or ortho-nitrophenol. Most preferably, the polymer is conjugated to the protein via an amide linkage formed from the para-nitrophenyl ester of the polymer. For example, the PEG-OH may be converted to PEG-O and reacted

with $BrCH_2CO_2CH_3$, the methyl ester may be hydrolyzed, and the acid may be reacted with p-nitrophenol in the presence of dicyclohexyl-carbodiimide to produce the activated polymer PEG-O-CH_2COO⟨O⟩NO_2. This polymer is, following purification, in turn reacted with available free amino group(s) on the antibiotic.

In another embodiment PEG-OH is reacted with a chloroformate (also called a chlorocarbonate) to form a PEG active ester in a single step. After the PEG active ester is formed, it is reacted with the antibiotic to form a PEG/antibiotic conjugate (see also Veronese et al., Biochem. and Biotech. 11:141-152 (1985). Chloroformates may be purchased from companies such as Aldrich. They may also be made as shown in equation (1).

$$Cl\text{-}\overset{\overset{O}{\|}}{C}\text{-}Cl + R\text{-}OH \rightarrow Cl\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R + HCl$$

The chloroformate is made by reacting phosgene, also known as carbonyl chloride, with an alcohol (R-OH) which contains electron withdrawing substituents on the carbon that carries the -OH. The alcohol is preferably an acidic alcohol, more preferably an acidic alcohol which contains aromatic rings which have high extinction coefficients. Examples of R groups are: N-hydroxy-succinimide, N-hydroxy-sulfosuccinimides, cyanomethyl alcohol, all nitro, chloro, and cyano substitutions on benzene, naphthalene, or larger aromatic ring systems which may or may not contain hetero-atoms, such as pyridine, para-nitrophenol (pNP), ortho-nitrophenol (oNP), etc. Most preferred R groups are pNP and oNP.

After the chloroformate is formed, it is reacted with PEG-OH to form a PEG active ester as shown in equation (2).

$$PEG\text{-}OH + Cl\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R \rightarrow PEG\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R + HCl$$

The chloroformate is reactive at two sites; at the bond between the chlorine and carbonyl group (more reactive) and the bond between the carbonyl and O-R group (less reactive). The more reactive site is where the chloroformate binds to the PEG. PEG-OH and the chloroformate are preferably added together at room temperature in an appropriate solvent, such as $CHCl_3$, or CH_2Cl_2. Preferably an acylation catalyst is added between 0 and 1 hours later, preferably the catalyst is pyridine or dimethyl pyridine. Preferably, the chloroformate is added up to 2-20 M excess, more preferably to a 15 M excess. The mixture is allowed to mix for preferably 4 hours, more preferably 16 hours. At this point, a precipitate may form. It is removed by filtration and discarded. Filtering devices such as Whatman glass fiber filters (GH/B) are acceptable. The resulting solution contains the PEG active ester as well as unreacted PEG and excess chloroformate. It is precipitated by adding an ether, preferably the

ether is diethyl ether. The precipitate contains the PEG active ester and can be washed with appropriate solvents such as ether, redissolved and reprecipitated if necessary.

After the PEG active ester is formed, it can be conjugated with an amino group (for example) on the antibiotic (AB) as shown in equation (3):

$$PEG\text{-}O\text{-}\overset{O}{\overset{\|}{C}}\text{-}O\text{-}R + NH_2\text{-}AB \rightarrow PEG\text{-}O\text{-}\overset{O}{\overset{\|}{C}}\text{-}NH\text{-}AB + ROH$$

Thus, a covalent bond between the PEG and the antibiotic is formed. In the final product, the PEG moiety is bound to the antibiotic by a

urethane, also called a carbamate, linkage having the structure $-O-\overset{O}{\overset{\|}{C}}-NH-$. This linkage is relatively stable and will keep PEG conjugated to the antibiotic with little or no hydrolysis under physiological conditions.

If the polymer is being conjugated to the antibiotic via a cysteine residue, a preferred mode of conjugation is as follows: mPEG-NH_2 as described above is reacted at room temperature for preferably 0.5-1.5 hours with N-maleimido-6-aminocaproic ester of 4-hydroxy-3-nitrobenzene sulfonic acid (mal-sac-HNSA), which is described by Nitecki et al., High-Technology Route to Virus Vaccines (Amer. Soc. for Microbiol., 1985), pp.43-46, supra. The latter reaction is preferably conducted with about a 5-fold molar excess of mal-sac HNSA over PEG-NH_2. After removal of hydrolysed or unreacted mal-sac HNSA (e.g., by dialysis, diafiltration, or size-exclusion chromatography), the PEG-maleimide (that has been formed) can then be reacted with the sulfhydryl group of the antibiotic at room temperature in a buffer using equimolar amounts of PEG-maleimide and antibiotic. Other bifunctional crosslinking reagents, such as N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), or $XCH_2CO\text{-}NH(CH_2)_5$-HNSA ester wherein X is Br, Cl, or I, can perform the same function as mal-sac HNSA under a variety of reaction conditions known to those skilled in the art.

Typically the activated polymer is conjugated with the antibiotic in the following concentrations. Between 0.12 and 2.50 mmole antibiotic are solubilized in a buffer, such as phosphate buffer, at a pH of between 5.0 and 8.0. The activated polymer is added to the solution to a concentration between 0.1 and 10.0 mmolar. The solution is allowed to react by stirring at room temperature for a period of at least 2 hours, more preferably at least 24 hours. It must be appreciated that the quantities of antibiotic and activated polymer will change if it is desired to scale up the present process to produce larger quantities of the polymer-antibiotic. It must also be appreciated that during scale up different reaction conditions and different reaction ratios may be preferred.

After the solution has been allowed to react, it contains the polymer-antibiotic conjugate, hydrolyzed polymer, the activated polymer, the antibiotic, the leaving group of the active ester, as well as other products. At this stage, the polymer-antibiotic conjugate must be separated and purified from the mixture. There are many separation general methods known to those of ordinary skill in the art which may accomplish this task. The present methods are explained below.

Purification, Uses, And Formulations

After the conjugation reaction, there can be a mixture of species, the identity of which will depend upon the reaction conditions. These species which differ in the number of PEG moieties conjugated to the antibiotics may be separated by various methods including chromatography (ie., HPLC) and electrophoresis. Hydrophobic interaction chromatography (HIC) using phenyl-Sepharose can be useful. Size separation may also be accomplished using molecular sieve chromatography. In addition, the polymer-antibiotic conjugates can be salted out using ammonium sulfate, $(NH_4)_2SO_4$; sodium sulfate, Na_2SO_4; magnesium salts and phosphates. The more highly conjugated species can precipitate at lower salt concentrations than the less conjugated and unconjugated species. Recycling of partially purified species through any of these techniques may result in a substantially better yields of polymer-antibiotic conjugates. Other example methods are size exclusion chromatography, ion exchange chromatography, affinity chromatography, high voltage paper electrophoresis, gel electrophoresis, and differential precipatation.

After a purified conjugate has been prepared, it may be tested for in vivo or in vitro activity. In vitro activity is useful as a preliminary screening mechanism and may be tested by a variety of different procedures. One procedure is to administer a sample of the conjugate, along with appropriate controls, to a lawn of potentially susceptible bacteria, fungi, or other target microorganisms. The plates can be read after overnight incubation at approximately 37° centigrade. Zones of clearing indicate that the microorganisms are inhibited by the conjugate. Preferably, the conjugate has substantially the same activity as the free antibiotic. "Substantially the same activity as the free antibiotic "is defined as providing a similar inhibitory effect in an assay, such as the method described above, wherein the effects are within a factor of twenty based on the molarity of the antibiotic. In vivo activity may be determined by administrating the conjugate to animals that are given a lethal bacterial, viral, or fungal infection and monitoring their survival. Polymer-antibiotic conjugates that are useful in the present invention are those that have a substantially greater theraputic index than that of the free antibiotic. The difference between theraputic index of the conjugate and the free

antibiotic can be tested in appropriate animal models by determining the ED_{50} and LD_{50}.

As mentioned above, antibiotics are effective to treat various disease states. Those disease states include bacterial, fungal, and protozoan infections of the urinary tract, gastrointestinal tract, and meninges, as well as septicemia, bacteremia, bacteriuria, ocular infections, and others. The present polymer-antibiotic conjugate can be used for the same indications. Furthermore, the present conjugate can have broader therapeutic utility than the original antibiotic because covalent conjugation may give it different properties. For example, conjugation may: change the specific activity; increase/decrease solubility; and change the clearance mechanism to ultimately decrease toxicity (the conjugate may reduce the dose needed for efficacy which would decrease the side effects).

To be used for therapeutic indications the present polymer-antibiotic conjugate may be incorporated into a pharmaceutical composition because it is considered therapeutically effective for human and veterinary uses. It can be formulated in a nontoxic, inert, pharmaceutically aqueous carrier medium, preferably at pH from 6 to 8. The polymer-antibiotic conjugate can be formulated with a number of excipients such as sugars, buffers, preservatives, amino acids, polymers, or other proteins. Specific examples include sucrose, fructose, dextrose, maltose, glucose, mannitol, sorbitol, inositol, galactitol, xylitol, lactose, trehalose, citrate, acetate, phosphate, Ringer's and Hank's solutions, cysteine, arginine, carnitine, alanine, glycine, lysine, valine, leucine, etc.

As a composition, it may be parenterally administered to subjects by the methods known in the art. This composition may contain other compounds that increase the effectiveness or promote the desirable qualities of the polymer-antibiotic conjugate. The composition must be safe for administration via the route that is chosen, sterile, and effective. Preferably, the formulation is administered to humans or animals in therapeutically effective amounts. Preferably, these amounts may be determined using in vivo efficacy data obtained after preclinical testing for the various indications.

The present process will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

Example I

PEG Active Ester Preparation

PEG was reacted with para-nitrophenyl chloroformate (pNP chloroformate) to produce an active PEG. The pNP chloroformate was obtained from Aldrich.

(A) 1.9 grams of PEG, having an average molecular weight of 1900 (purchased from Aldrich), was dissolved in approximately 13 ml of $CHCl_3$, and 1 gram of pNP chloroformate was added. The resulting solution was clear. After approximately 2 to 3 hours at room temperature, 1.5 ml of pyridine was added and a precipitate formed. The precipitate was removed by filtration using a Whatman glass microfiber filter (GF/B) and discarded. Approximately 200 ml of diethyl ether was added to the $CHCl_3$ solution. A large gelatinous precipitate formed which was filtered after 1 hour and washed with ether. The precipitate was dried to remove the ether. It contained the active PEG.

(B) 2.5 grams of PEG, having an average molecular weight of 5,000 (purchased from Aldrich), were dissolved in approximately 13 ml of $CHCl_3$. One gram of pNP chloroformate was added and the procedure noted above was repeated.

Example 2

Conjugation of PEG to Polymyxin

360 mg (0.3 mmole) polymyxin (purchased from Sigma) was dissolved in 15 ml 0.1 M PO_4 at pH 7.5. Because the pH of the resulting solution was 7.35 it was titrated to 7.5 with 0.1 M NaOH. Active PEG was prepared by a method similar to Example 1 using 4,000 mw PEG. 2.4 g (0.6 mmole) of the active PEG was added to the polymyxin solution at room temperature while stirring. The pH dropped to 4.96 and was readjusted to 7.5 with 0.1 M NaOH. The solution was stirred for 45 minutes at which time it was determined that only 15% of the activated PEG remained from the initial 83%.

Example 3

Purification of PEG-Polymyxin

The solution from Example 2 was purified by first separating it on a G-50 Sephadex size exclusion column equilibrated with either glass distilled water (0.1 N acetic acid has been used). The viscosity of the solution was lowered by dilution with eluent to avoid damaging the column. Fractions were eluted and monitored spectrophotometrically, see Figure 1. The largest molecules co-eluted with the void volume in fractions 30 through 60; they were the PEG-polymyxin conjugates and the

free PEG. Free polymyxin, unreacted active PEG, and pNP eluted in later fractions (70 through 110) and separated well from the larger molecules. However, some pNP may have eluted with the PEG as it appeared in the second purification step below where it was removed. Fractions 30 through 60 were further separated as described below.

An ion-exchange column of S-Sepharose was equilibrated with 0.01 M phosphate buffer at pH 6.0. The mixture of PEG, PEG-polymyxin, and residual pNP (fractions 30-60) was applied to the column in same 0.01 M phosphate buffer at pH 6.0. After the uncharged, free PEG and pNP were eluted (fractions 0 through 20), a linear NaCl gradient formed by 500 ml 0.5 M NaCl in 0.01 M phosphate at pH 6.0 run into 500 ml 0.01 M phosphate at pH 6. 0 (at fraction 25)was used and the fractions were evaluated spectrophotometrically. A second gradient was run into the sample at fraction 258 to ensure that all the molecular species were removed. The second gradient comprised 500 ml of 1.0 M NaCl in 0.01 M phosphate buffer at pH 6, and is known to elute unmodified polymyxin from S-Sepharoese. Several peaks were resolved (see Fig. 2). These peaks were desalted on a G-50 Sephadex column because dialysis was insufficient to remove salt to a concentration which showed biological activity.

Example 4

PEG-Polymyxin Activity

This experiment was run to determine if the PEG-polymyxin conjugate inhibited the growth of E. coli, strain SM18.

A lawn of E. coli SM18 was plated on tryptic soy agar (TSA) plates at a concentration of 10^8 organisms per plate. Multiple plates were made to test the inhibitory effect of the PEG/polymyxin conjugate against controls for polymyxin alone, PEG alone, and sterile saline. The polymyxin was polymyxin B obtained from Sigma having 7,900 USP units per milligram. The PEG had a molecular weight of 5,000 and was similar to the PEG used in Example 1. The PEG-polymyxin conjugate was made and purified as shown in Examples 1 through 3. Solutions containing 100 mg/ml of polymyxin and PEG were made in sterile water. 10 microliters of each solution (polymyxin, PEG, PEG-polymyxin, and saline) were placed onto the TSA plate. The plates were incubated at 37° centigrade and were checked for zones of clearing after approximately 18 to 24 hours.

The results show that both polymyxin and the PEG-polymyxin conjugate (fractions 100 to 180 in Fig. 2) created zones of clearing whereas the PEG and saline

controls did not (also, fractions 40 to 80 of Fig. 2 did not exhibit activity). It is evident that the polymer-polymyxin conjugate retained the effectiveness of unconjugated polymyxin and inhibited bacterial growth. It was also evident that the PEG-polymyxin conjugate retained substantially the same activity as free polymyxin.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

We Claim

1. A biologically active polymer-antibiotic conjugate comprising at least one biologically acceptible polymer conjugated to one antibiotic molecule through a covalent bond that is stable for a substantial period of time, wherein the therapeutic index of the conjugate is substantially greater than that of the free antibiotic and the activity of the conjugate is substantially the same as the free antibiotic.

2. A conjugate in accordance with Claim 1, wherein the polymer is selected from the group consisting essentially of polyalkylene glycols, copolymers of different polyalkylene glycols, polyvinylpyrrolidone, polyvinyl alcohol, homopolymers of amino acids, cellulose and cellulose derivatives, sulfonated sugars, starch, and starch derivatives.

3. A conjugate in accordance with Claim 2, wherein the polymer is selected from the group consisting essentially of polyethylene glycol, polyoxyethylated glycerol, a co-polymer of propylene glycol and ethylene glycol, heparin, and polyproline.

4. A conjugate in accordance with Claim 1, wherein the polymer is polyethylene glycol.

5. A conjugate in accordance with Claim 1, wherein the antibiotic has a short circulating half-life or is not readily water soluble and is selected from the group consisting essentially of: aminoglycosides; ß-lactams; chloramphenicol and its derivatives; lincosaminides; macrolides; nucleosides; oligosaccharides; peptides; polyenes; tetracyclines; sulfonamides; nitrofurans; and quinolone carboxylic acids.

6. A conjugate in accordance with Claim 5, wherein the antibiotic is selected from the group consisting essentially of: peptides; polyenes; aminoglycosides; and tetracyclines.

7. A conjugate in accordance with Claim 1, wherein the antibiotic is selected from the group consisting essentially of polymyxin and amphotericin.

8. A conjugate in accordance with Claim 1, wherein the antibiotic is polymyxin.

9. A conjugate in accordance with Claim 1, wherein the polymer has molecular weight between 600 and 20,000.

10. A conjugate in accordance with Claim 1, wherein the polymer has molecular weight between 2,000 and 12,000.

11. A conjugate in accordance with Claim 1, wherein the antibiotic is amphotericin.

12. A conjugate in accordance with Claim 1, wherein the conjugate is formulated with pharmaceutically acceptable excipients.

13. A conjugate in accordance with Claim 12, wherein the excipients are selected from the group consisting essentially of buffers, sugars, polyols, and polymers.

14. A biologically effective polymer-antibiotic conjugate comprising PEG covalently conjugated to polymyxin.

15. A conjugate in accordance with Claim 14, wherein PEG is unsubstituted or substituted on one end with an alkyl group having 1 to 3 carbons.

16. A conjugate in accordance with Claim 15, wherein PEG has a molecular weight between 600 and 20,000.

17. A conjugate in accordance with Claim 16, wherein PEG has a molecular weight between 2,000 and 12,000.

18. A conjugate in accordance with Claim 17 , wherein PEG is covalently conjugated to polymyxin by a urethane, an amide, or an ester bond.

19. A conjugate in accordance with Claim 18 further comprising formulating the conjugate with pharmaceutically acceptable excipients.

20. A conjugate in accordance with Claim 19, wherein the excipients are selected from the group consisting essentially of buffers, sugars, polyols, and polymers.

21. A process for treating a bacterial, fungal, or other infectious disease comprising administering a theraputically effective amount of a biologically active polymer-antibiotic conjugate comprising at least one biologically acceptible polymer conjugated to one antibiotic molecule through a covalent bond that is stable for a substantial period of time, wherein the theraputic index of the conjugate is substantially greater than that of the free antibiotic and the activity of the conjugate is substantially the same as the free antibiotic.

22. A process for treating a bacterial, fungal, or other infectious disease in accordance with Claim 21 wherein the conjugate comprises PEG and the antibiotic is selected from the group consisting essentially of polymyxin and amphotericin.

23. A process for binding lipopolysaccharide in a patient suffering from septic shock comprising administering a theraputically effective amount of a polymer-polymyxin conjugate.

Figure 1

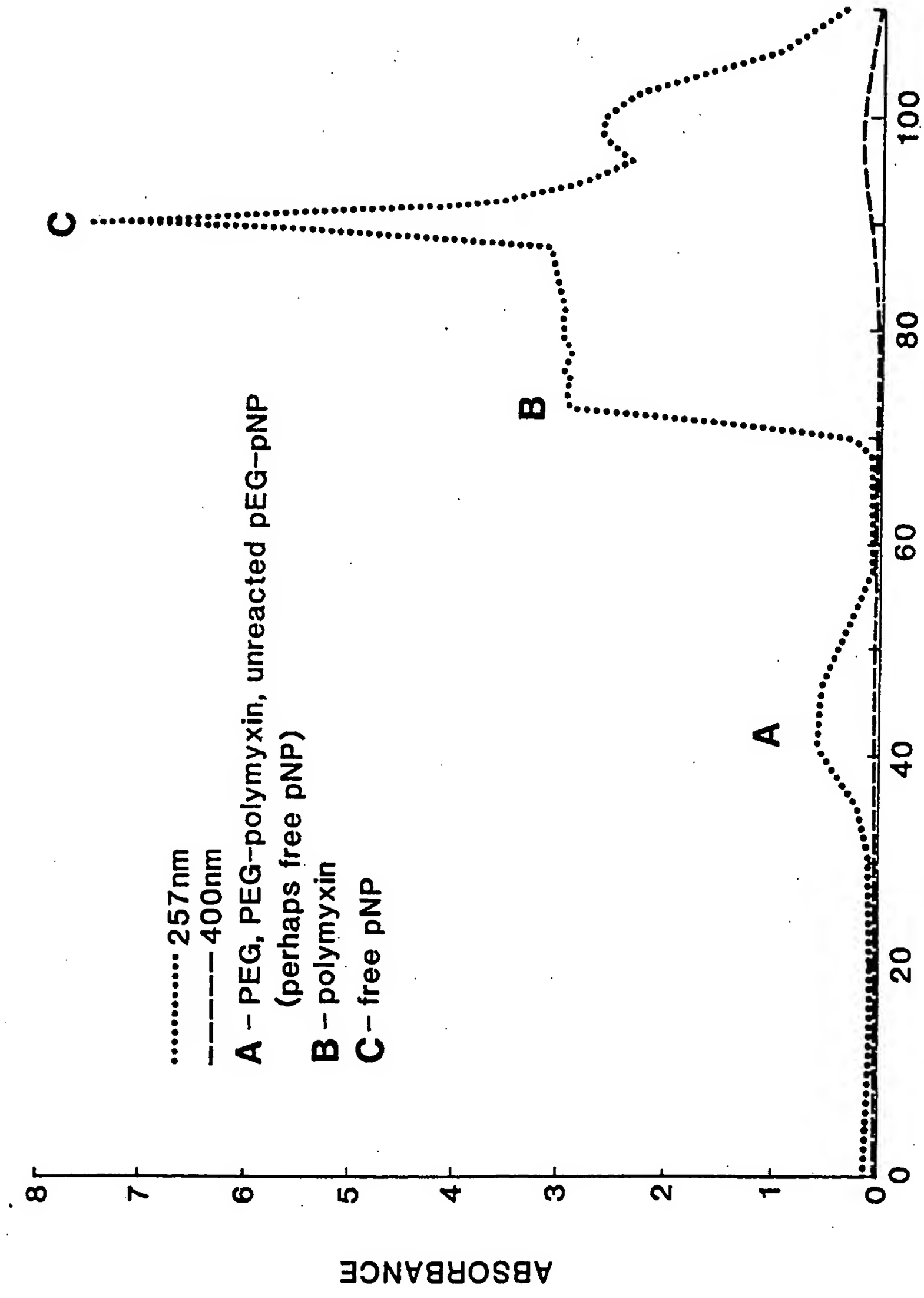
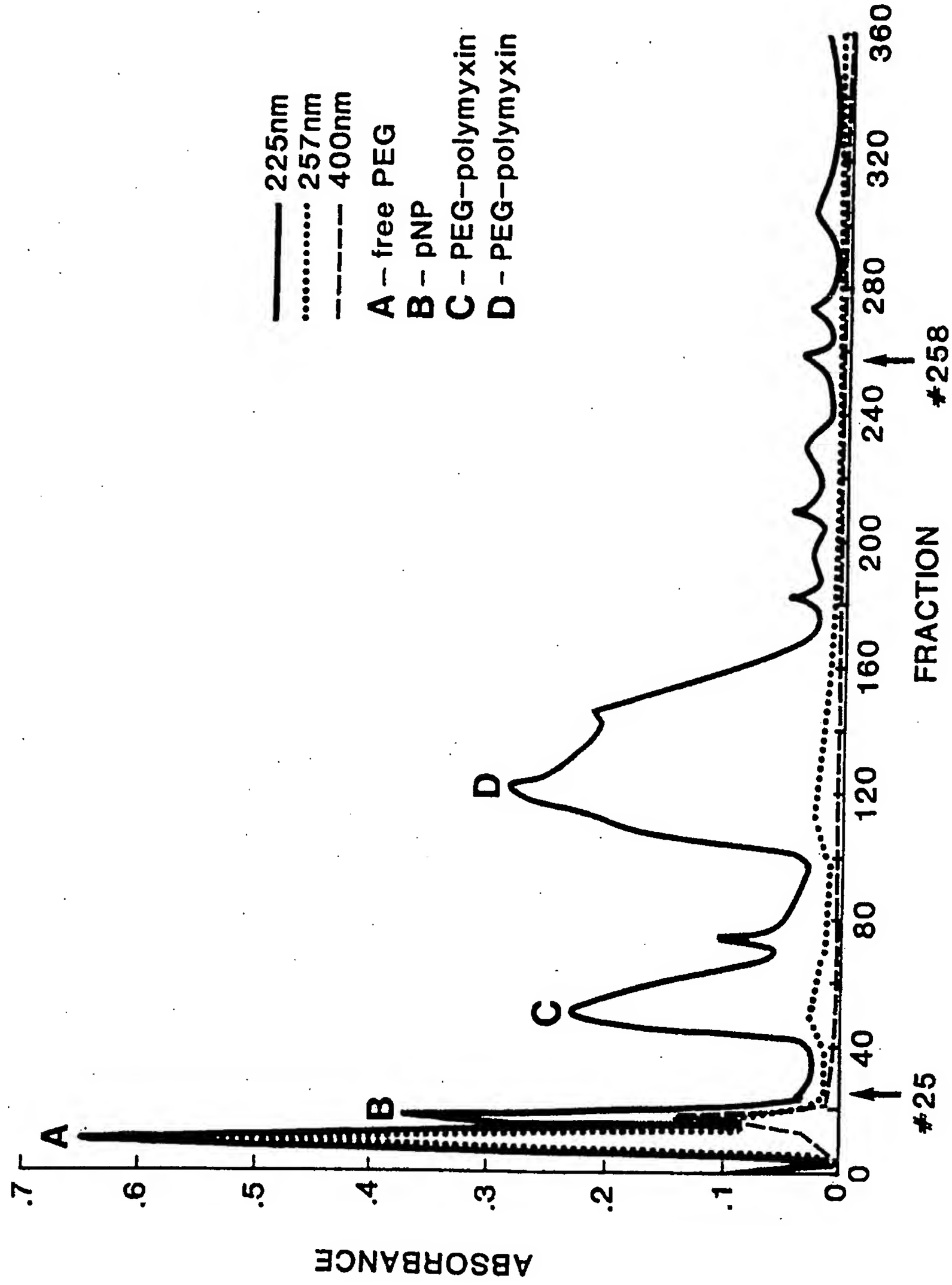

ABSORBANCE
0
1
2
3
4
5
6
7
8
FRACTION
20
40
60
80
100
A
B
C
A – PEG, PEG–polymyxin, unreacted pEG–pNP
(perhaps free pNP)
B – polymyxin
C – free pNP
257nm
400nm

Figure 2


—— 225nm
..... 257nm
– – – 400nm
A – free PEG
B – pNP
C – PEG–polymyxin
D – PEG–polymyxin
ABSORBANCE
.7
.6
.5
.4
.3
.2
.1
0
FRACTION
0
40
80
120
160
200
240
280
320
360
#25
#258
A
B
C
D

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/03252

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 47/48

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Makromol. Chem., vol. 180, 1979, V. Hofmann et al.: "Pharmakologisch-aktive polymere, 19[a)] polymere mit kovalent gebundenen Streptomycin-sulfat", pages 837-841, see the whole article --	1,5-6,12-13, 19-20
X	STN File Server, Chemical Abstracts, vol. 100, (Columbus, Ohio, US), S. Zalipsky et al.: "Attachment of drugs to polyethylene glycols", see abstract 197692q, & Eur. Polym. J., 19(12), 1177-83 (cited in the application) --	1-5,5-6, 12-13,19-20
X,Y	FR, A, 2342740 (PHARMACO, INC.) 30 September 1977 ./.	1-20

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9th October 1990	14. 11. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalie Weinberg

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	see page 4, lines 17-23; page 6, line 26 - page 18, line 3; page 20, line 21; claim --	
X	Polymer Bulletin, vol. 16, no. 4, October 1986, Springer-Verlag, Berlin, DE , C.I. Simionescu et al.: "Bioactive polymers. XLII. Coupling of ampicilline on (chlorocarbonyl-methyl)cellulose", pages 319-325, see the whole article --	1-2,5-6, 12-13,19-20
P,X	Biomaterials, vol. 10, no. 9, November 1989, (Guildford, Surrey, GB), C. Beldie et al.: "Bioactive polymers. LX. Kinetics of delayed release neomycin-xanthan complex", pages 622-624, see the whole article --	1-2,5-6, 12-13,19-20
Y	WO, A, 87/00056 (CETUS CORPORATION) 15 January 1987 see claims ----------	1-20

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers ...**..., because they relate to subject matter not required to be searched by this Authority, namely:

** Claim numbers 21-23

See Rule 39.1 (iv): methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9003252 SA 38081

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/10/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
FR-A- 2342740	30-09-77	AU-A-	2271177	31-08-78
		DE-A-	2709059	15-09-77
		JP-A-	52108016	10-09-77
		NL-A-	7702228	06-09-77
		SE-A-	7702261	07-09-77
WO-A- 8700056	15-01-87	AU-A-	5970086	30-01-87
		EP-A-	0229108	22-07-87
		JP-T-	62503171	17-12-87
		US-A-	4917888	17-04-90
		US-A-	4766106	23-08-88